New 2,4,5-triarylimidazoles based on a phenylalanine core: synthesis, photophysical characterization and evaluation as fluorimetric chemosensors for ion recognition

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Abstract: Novel fluorescent 4,5-diarylimidazolyl-phenylalanines **3a**-**d** were prepared by reaction of *N*-*tert*-butyloxycarbonyl-4-formylphenylalanine methyl ester and appropriate (hetero)aromatic diones. The photophysical properties of these new unnatural amino acids were evaluated by UV-Vis absorption and fluorescence spectroscopy in solvents of different character and aqueous mixtures with acetonitrile. They were evaluated as novel amino acid based fluorimetric chemosensors for ions through spectrophotometric and spectrofluorimetric titrations with biologically and analytically important anions and cations such as F^-, OH^-, Cu^{2+} and Fe^{3+}. The results indicate that there was a strong interaction through the donor N, O and S atoms at the side chain, especially for 4,5-di(furan-2'-yl)imidazolyl-phenylalanine **3a** and 4,5-di(thiophen-2'-yl)imidazolyl-phenylalanine **3b** with Cu^{2+} and Fe^{3+}, in a 1:1 complex stoichiometry. The photophysical and metal ion sensing properties of these amino acids suggest that they can be suitable for incorporation into chemosensory peptidic frameworks.

1. Introduction

Optical sensing based on colorimetric and fluorimetric probes is a very appealing topic of research, considering the large number of chromo/fluorophores available and the simple instrumentation required (when using colorimetric probes), and the low detection limits (when using fluorescence probes) [1]. Hence, the design of novel organic fluorophores that can be used in biomolecule labelling and chemosensing of organic and inorganic molecules involved in biological pathways is worth exploring [2]. The development of probes containing binding sites capable of sensing anions and cations, preferably in aqueous media, is a very active area within the chemical sensing field [3]. Imidazole derivatives are known as neutral binding groups for anions [4], and the coordination ability of the imidazole group depends on the acidity of the NH proton that can be modulated by suitable substituents such

as heteroaromatic rings like thiophene, pyrrole or furan. On the other hand, the two nitrogen atoms in imidazole enable the coordination of metal cations by this five-membered heterocycle [5]. 2,4,5-Triarylimidazoles have found application in materials sciences for their interesting optoelectronic properties that can be tuned by careful selection of substituents at positions 2, 4 and 5: replacement of the aryl group by an heterocyclic group results in larger π-conjugated systems with improved optical properties for application in two-photon absorption applications, two-photon fluorescence microscopy, high density storage and 3D microfabrication, nonlinear optics, OLEDs, and chemosensors [6]. 2,4,5-Triarylimidazoles have also been applied in medicinal chemistry, for example as ligands for Ru(II) and Pt(II) complexes, as probes of DNA structure or new therapeutic agents due to their capacity to bind or interact with DNA [7].

Synthetically modified amino acids are useful for the preparation of intrinsically labelled peptides with tailored properties such as increased fluorescence, conformational rigidity, and metal complexation ability, among others. Recent examples in the literature refer the use of fluorescent unnatural amino acids in studies of molecular flexibility and protein folding, substrate binding activity of proteins, antigenicity or enzymatic activity, targeting peptides for molecular imaging, peptidomimetics biological activity and protein engineering [8]. Metallic cations can be complexed through N, O and S donor atoms in amino acids, at the main and side chains, and in aromatic heterocycles, whereas anion coordination, based on hydrogen bonding and electrostatic interactions, can arise from amino acid side and main chain OH and NH groups, or from NH groups in heterocycles [9]. Therefore, the insertion of suitable heterocyclic systems at the side chain of natural amino acids can add extra functionality to the resulting amino acid, bearing a metal ion chelating site for stable complex formation and subsequent incorporation into a peptide.

Given these facts, our current research interests include the synthesis and characterization of unnatural amino acids bearing fluorescent oxygen, nitrogen and sulphur heterocycles [10], imidazole and benz-X-azole derivatives with interesting optical properties [11] and innovative heterocyclic colorimetric/fluorimetric chemosensors for anions and cations based on amino acid moieties [12]. To the best of our knowledge, this is the first report on the synthesis of 2,4,5-triaryl-imidazole derivatives combined with an amino acid core, the evaluation of the photophysical properties in different solvents and the chemosensing ability in the presence of anions and cations.

2. Experimental

2.1. Synthesis general

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer using KBr discs. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 ºC. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference. Assignments were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Low and high resolution mass spectra were obtained at ''C.A.C.T.I. Unidad de Espectrometria de Masas'' at the University of Vigo, Spain. Commercially available reagents **2a-d** were purchased from Sigma–Aldrich, ACROS, or TCI and used as received. Compound **1** was synthesised as reported elsewhere [13].

2.2. General procedure for the synthesis of imidazolyl-phenylalanines 3a-d

N-tert-Butyloxycarbonyl-4-formylphenylalanine methyl ester **1** (1 equiv) and the appropriate dione **2** (1 equiv) were dissolved in acetic acid (5 mL/mmol) in the presence of ammonium acetate (20 equiv) and heated at reflux for 2 hours. After cooling, the mixture was poured over crushed ice and extracted with ethyl acetate (3 x 5 mL). After drying the organic layer over anhydrous $MgSO_4$, the solvent was removed in a rotary evaporator and a solid was obtained. The crude solid was purified by column chromatography, using mixtures of dichloromethane and methanol of increasing polarity as eluent.

2.2.1. *N*-acetyl-4-(4',5'-di(furan-2''-yl)imidazol-2'-yl) phenylalanine methyl ester (3a). Starting from aldehyde **1** (0.050 g, 0.163×10^{-3} mol) and 1,2-di(furan-2-yl)ethane-1,2-dione **2a** (0.031 g, 0.163×10^{-3} mol), compound **3a** was isolated as an orange solid (0.035 g, 0.084×10^{-3} mol, 42%). mp = 209.8-210.7 ºC. IR (KBr 1%, cm^{-1}): ν = 3340, 3078, 2928, 1737, 1691, 1633, 1596, 1526, 1518, 1437, 1389, 1368, 1322, 1291, 1256, 1220, 1166, 1109, 1086, 1059, 1029, 994, 971, 920, 854, 813, 796, 772, 684. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.99 (s, 3H, CH_3 Ac), 3.06-3.20 (m, 2H, β-CH_2), 3.72 (s, 3H, OCH_3), 4.85-4.88 (m, 1H, α-H),

6.11 (d, *J* 7.6 Hz, 1H, NH Ac), 6.53 (dd, *J* 3.6 and 1.8 Hz, 2H, 2 × H4''), 6.99 (d, *J* 3.6 Hz, 2H, 2 × H3''), 7.14 (d, *J* 8.0 Hz, 2H, H2 and H6), 7.50 (d, *J* 1.8 Hz, 2H, 2 × H5''), 7.84 (d, *J* 8.0 Hz, 2H, H3 and H5). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 23.07 (CH_3 Ac), 37.72 (β-CH_2), 52.43 (OCH_3), 53.16 (α-C), 107.80 (C3''), 111.76 (C4''), 125.81 (C3 and C5), 127.24 (C4' or C5'), 127.87 (C4'or C5'), 129.69 (C2 and C6), 130.28 (C4), 137.20 (C1), 141.54 (C5''), 145.83 (C2') 155.33 (C2''), 169.89 (C=O Ac), 171.90 (C=O ester). UV/Vis (ethanol, nm): λ_{max} (log ε) = 317 (4.21). MS *m/z* (ESI, %): 420 ([M+H]$^+$, 100). HRMS: *m/z* (ESI) calcd for $C_{23}H_{22}N_3O_5$ 420.15606; found 420.15508.

2.2.2. *N*-acetyl-4-(4',5'-di(thiophen-2''-yl)imidazol-2'-yl) phenylalanine methyl ester (3b). Starting from aldehyde **1** (0.050 g, 0.162 × 10^{-3} mol) and 1,2-di(thiophen-2-yl)ethane-1,2-dione **2b** (0.036 g, 0.162 × 10^{-3} mol), compound **3b** was isolated as a yellow solid (0.038 g, 0.084 × 10^{-3} mol, 45%). mp = 215.9-217.0 ºC. IR (KBr 1%, cm^{-1}): ν = 3431, 3146, 2977, 1711, 1655, 1596, 1534, 1513, 1446, 1419, 1391, 1366, 1332, 1259, 1195, 1168, 1120, 1060, 1020, 994, 959, 939, 912, 892, 871, 817, 735, 695, 646, 613. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.79 (s, 3H, CH_3 Ac), 2.88-3.07 (m, 2H, β-CH_2), 3.59 (s, 3H, OCH_3), 4.47-4.52 (m, 1H, α-H), 7.00 (dd, *J* 5.2 and 3.6 Hz, 1H, H4''), 7.15 (dd, *J* 3.6 and 0.8 Hz, 1H, H3''), 7.20 (dd, *J* 4.2 and 3.6 Hz, 2H, H4'''), 7.31 (d, *J* 8.4 Hz, 2H, H2 and H6), 7.40-7.42 (m, 2H, H5'' and H3'''), 7.69 (d, *J* 5.2 and 1.2 Hz, 1H, H5'''), 7.93 (d, *J* 8.4 Hz, 2H, H3 and H5), 8.36 (d, *J* 8.0 Hz, 1H, NH Ac), 12.80 (br s, 1H, NH). ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 22.23 (CH_3 Ac), 36.57 (β-CH_2), 51.84 (OCH_3), 53.43 (α-C), 120.60 (C4' or C5'), 123.39 (C3''), 124.84 (C3'''), 125.23 (C3 and C5), 127.31 (C4''), 127.40 (C5'''), 127.59 (C4'''), 128.09 (C4), 128.41 (C5''), 129.43 (C2 and C6), 130.79 (C2'''), 133.55 (C4' or C5'), 137.45 (C2''), 137.83 (C1), 145.66 (C2'), 169.35 (C=O Ac), 172.13 (C=O ester). UV/Vis (ethanol, nm): λ_{max} (log ε) = 313 (4.21). MS *m/z* (ESI, %): 452 ([M+H]$^+$, 100). HRMS: *m/z* (ESI) calcd for $C_{23}H_{22}N_3O_3S_2$ 452.10911; found 452.10971.

2.2.3. *N*-acetyl-4-(4',5'-diphenyl-imidazol-2'-yl) phenylalanine methyl ester (3c). Starting from aldehyde **1** (0.049 g, 0.160 × 10^{-3} mol) and 1,2-diphenylethane-1,2-dione **2c** (0.033 g, 0.160 × 10^{-3} mol), compound **3c** was isolated as an orange solid (0.025 g, 0.057 × 10^{-3} mol, 44%). mp = 203.1-204.2 ºC. IR (KBr 1%, cm^{-1}): ν = 3320, 3178, 3028, 1837, 1751, 1633, 1596, 1525, 1418, 1382, 1368, 1300, 1290, 1254, 1222, 1166, 1100, 1085, 1059, 1029, 994,

970, 923, 855, 812, 796, 772, 683. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.80 (s, 3H, CH_3 Ac), 2.88-3.07 (m, 2H, β-CH_2), 3.60 (s, 3H, OCH_3), 4.47-4.52 (m, 1H, α-H), 7.19-7.49 (m, 8H, 2xPh), 7.48 (d, *J* 7.8 Hz, 2H, H2 and H6), 7.98 (d, *J* 7.8 Hz, 2H, H3 and H5), 8.36 (d, *J* 7.6 Hz, 1H, NH Ac), 12.62 (br s, 1H, NH). ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 22.23 (CH_3 Ac), 36.58 (β-CH_2), 51.83 (OCH_3), 53.47 (α-C), 125.11 (C3 and C5), 126.50 (C4''), 127.05 (C3'' and C5''), 127.69 (C4' or C5'), 127.74 (C4'''), 128.17 (C2'' and C6''), 128.39 (C2 and C6), 128.65 (C3''' and C5'''), 128.71 (C4), 129.36 (C2''' and C6'''), 131.07 (C1''), 135.17 (1'''), 137.03 (C4' or C5'), 137.40 (C1), 145.44 (C2'), 169.35 (C=O Ac), 172.16 (C=O ester). UV/Vis (ethanol, nm): λ_{max} (log ε) = 304 (4.28). MS *m/z* (ESI, %): 440 ($[M+H]^+$, 100). HRMS: *m/z* (ESI) calcd for $C_{27}H_{26}N_3O_3$ 440.19687; found 440.19628.

2.2.4. *N*-acetyl-4-(4',5'-di(pyridin-2''-yl)imidazol-2'-yl) phenylalanine methyl ester (3d). Starting from aldehyde **1** (0.020 g, 0.066 × 10^{-3} mol) and 1,2-di(pyridin-2-yl)ethane-1,2-dione **2d** (0.014 g, 0.066 × 10^{-3} mol), compound **3d** was isolated as an orange solid (0.020 g, 0.045 × 10^{-3} mol, 44%). mp = 123.9-124.7 ºC. IR (KBr 1%, cm^{-1}): ν = 3351, 3068, 2918, 1764, 1691, 1632, 1526, 1509, 1437, 1365, 1320, 1293, 1256, 1166, 1109, 1086, 1049, 1019, 991, 920, 853, 812, 796, 772, 684. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.96 (s, 3H, CH_3 Ac), 3.04-3.17 (m, 2H, β-CH_2), 3.69 (s, 3H, OCH_3), 4.83-4.88 (m, 1H, α-H), 6.04 (d, *J* 7.6 Hz, 1H, NH Ac), 7.11-7.14 (m, 2H, 2 × H5''), 7.13 (d, *J* 8.0, 2H, H2 and H6), 7.72 (t, *J* 7.3 Hz, 2H, 2 × H4''), 8.02 (d, *J* 7.3 Hz, 2H, 2 × H3''), 8.44-8.46 (m, 2H, H3 and H5), 8.64 (d, *J* 4.0 Hz, 2H, 2 × H6''). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 23.16 (CH_3 Ac), 37.79 (β-CH_2), 52.44 (OCH_3), 53.14 (α-C), 122.59 (C3''), 123.37 (C3 and C5), 126.46 (C5''), 127.88 (C4), 127.95 (C4' and C5'), 129.64 (C2 and C6), 137.38 (C1), 138.52 (C4''), 146.64 (C6''), 147.32 (C2''), 149.13 (C2'), 169.75 (C=O Ac), 171.91 (C=O ester). UV/Vis (ethanol, nm): λ_{max} (log ε) = 307 (4.25). MS *m/z* (ESI, %): 442 ($[M+H]^+$, 100). HRMS: *m/z* (ESI) calcd for $C_{25}H_{24}N_5O_3$ 442.18737; found 442.18648.

2.3. Spectrophotometric and spectrofluorimetric titrations and chemosensing studies for imidazolyl-phenylalanines 3a-d

UV–visible absorption spectra (200–700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. Organic solvents used in the spectroscopic studies were of spectroscopic grade. Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene

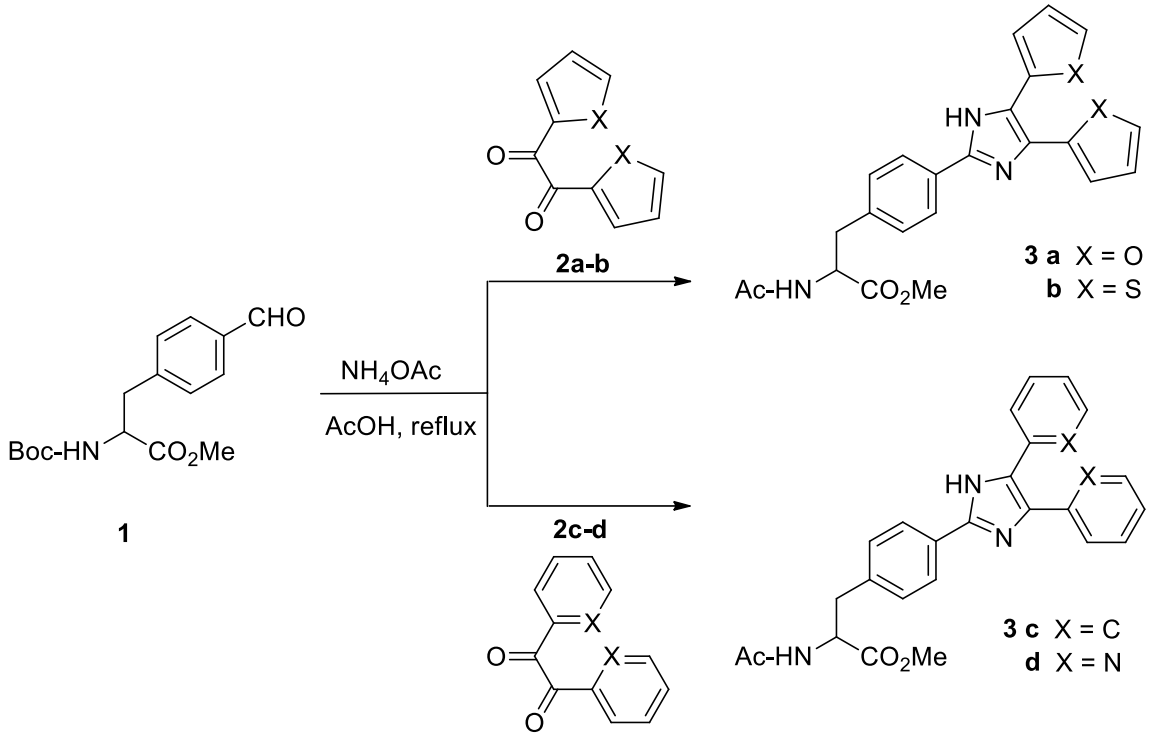
as standard (Φ_F = 0.95 in ethanol) [14]. The linearity of the absorption versus concentration was checked within the used concentration.

Solutions of imidazolyl-phenylalanines **3a-d** (ca. 1.0×10^{-5} to 1.0×10^{-6} M) and of the ions under study (ca. 1.0×10^{-1} to 1.0×10^{-3} M) (in the form of hydrated tetrafluorborate salts for Cu^+, Ag^+, Pd^{2+} and Co^{2+}, hydrated perchlorate salts for K^+, Cd^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Cr^{3+}, Cu^{2+}, Ni^{2+}, Cs^+, Na^+, Hg^{2+}, Pb^{2+}, Zn^{2+} and hydrated tetrabutylammonium salts for CH_3COO^-, F^-, Cl^-, Br^-, I^-, ClO_4^-, CN^-, NO_3^-, BzO^- and OH^-) were prepared in UV-grade acetonitrile or acetonitrile/water (80:20). Titration of the compounds with the several ions was performed by the sequential addition of ion stock solution to the phenylalanine solution, in a 10 mm path length quartz cuvette and emission spectra were measured by excitation at the wavelength of maximum absorption for each compound, with a 2 nm slit. The association constants and the binding stoichiometry were obtained with HypSpec software.

3. Results and discussion

3.1. Synthesis of 4,5-diarylimidazolyl-phenylalanines 3a-d

Novel 4,5-diarylimidazolyl-phenylalanines **3a-d** were synthesised by reaction of *N-tert*-butyloxycarbonyl-4-formylphenylalanine methyl ester **1** with the appropriate aromatic dione **2a-d**, through a Radziszewski reaction in the presence of ammonium acetate in acetic acid at reflux for 2 hours [15]. The pure compounds were isolated, after chromatography, in moderate yield (42-45%) and were characterized by the usual spectroscopic techniques. The acidic reaction media yielded the *N*-acetylated form of the amino acids, by substitution of the starting *N-tert*-butyloxycarbonyl (Boc) group (Scheme 1, Table 1). The synthesized amino acids are L-amino acids as the precursor **1** had L-configuration and no evidence was found for the loss of the integrity of the chiral centre in these reaction conditions by NMR.



Scheme. Synthesis of 4,5-diarylimidazolyl-phenylalanines **3a-d**.

Table 1. Yields, UV-visible absorption and fluorescence data for 4,5-diarylimidazolyl-phenylalanines **3a-d** in absolute ethanol ([**3a-d**]= 10^{-6}-10^{-5} M).

Compound	Yield (%)	UV/Vis		Fluorescence			
		λ_{abs} (nm)	log ε	λ_{em} (nm)	Stokes' shift (nm)	Stokes' shift (cm^{-1})	Φ_F
3a	42	317	4.21	405	88	6854	0.72
3b	45	313	4.21	420	107	8139	0.26
3c	44	304	4.28	382	78	6717	0.42
3d	44	307	4.25	426	119	9099	0.14

3.2. Photophysical study of 4,5-diarylimidazolyl-phenylalanines 3a-d

The photophysical properties of phenylalanines **3a-d** were evaluated and the UV-vis absorption and fluorescence spectra of degassed 10^{-6}-10^{-5} M solutions in absolute ethanol were measured (Table 1). Compounds **3a-d** displayed absorption and emission maxima at longer wavelengths (λ_{abs} and λ_{em}, respectively), when compared with the unsubstituted phenylalanine in the same solvent (λ_{abs} = 258 nm and λ_{em}= 280 nm), a fact related to the

nature of the pendant groups which resulted in extended intramolecular electron delocalization and a higher push-pull character of the whole system. In fact, the position of the red shifted absorption bands were clearly dependent on the electron donor strength of the (hetero) aromatic group at positions 4 and 5 of the imidazole: for **3a**, bearing a phenyl group (the least electron donor), the absorption band was centred at 304 nm. On changing to electron-deficient pyridyl-derivative (**3d**), the absorption maximum suffered a very slight bathochromic shift to 307 nm, whereas when heterocyclic electron donors such as furan (**3a**) or thiophene (**3b**) were present, the red shift was larger to 317 and 313 nm, respectively. 4,5-Diarylimidazolyl-phenylalanines **3a-d** showed modest to high relative fluorescence quantum yields, between 0.14 (**3d**) and 0.72 (**3a**), and displayed large Stokes' shifts. A large Stokes' shift is an interesting characteristic for a fluorescent probe that allows an improved separation of the light inherent to the matrix and the light dispersed by the sample.

Considering the results obtained in ethanol, the photophysical properties of phenyalanines **3a-d** were evaluated in other organic solvents of different character. The solvents tested were acetonitrile, dimethylsulfoxide, dichloromethane and diethyl ether, as examples of solvents with different polarity and proticity, with π* values by Kamlet and Taft [16]. The collected data revealed similar wavelengths of maximum absorption and emission and the fluorescence quantum yield did not vary significantly (Table 2). The overall trend revealed that phenylalanine **3a** with furyl pendants was the most fluorescent (with Φ_F in the range 0.64-0.77) whereas phenylalanine **3b** bearing thienyl pendants displayed the largest Stokes' shifts (between 105-109 nm).

Table 2. UV-visible absorption and emission data for 4,5-diarylimidazolyl-phenylalanines **3a-d** in organic solvents of different character, with π* values by Kamlet and Taft [16].

Cpd.	Solvent (π*)	UV/Vis		Fluorescence			
		λ_{abs}	log ε	λ_{em}	Stokes' shift (nm)	Stokes' shift (cm^{-1})	Φ_F
3a	Diethyl ether (0.27)	313	4.23	401	88	7011	0.76
	EtOH (0.54)	317	4.21	405	88	6854	0.72
	ACN (0.75)	317	4.24	413	96	7333	0.77
	DCM (0.82)	324	4.21	410	86	6474	0.64

	DMSO (1.00)	317	4.26	409	92	7096	0.64
	Diethyl ether (0.27)	312	4.23	419	107	8185	0.29
	EtOH (0.54)	313	4.21	420	107	8139	0.26
3b	ACN (0.75)	313	4.28	422	109	8252	0.30
	DCM (0.82)	318	4.25	423	105	7806	0.26
	DMSO (1.00)	320	4.29	428	108	7886	0.14
	Diethyl ether (0.27)	309	4.27	383	74	6253	0.44
	EtOH (0.54)	304	4.28	382	78	6717	0.42
3c	ACN (0.75)	305	4.30	383	78	6677	0.46
	DCM (0.82)	309	4.27	383	74	6253	0.43
	DMSO (1.00)	312	4.31	391	79	6476	0.20
	Diethyl ether (0.27)	306	4.25	388	82	6907	0.10
	EtOH (0.54)	307	4.25	426	119	9099	0.14
3d	ACN (0.75)	305	4.25	398	93	7661	0.14
	DCM (0.82)	307	4.25	394	87	7193	0.14
	DMSO (1.00)	310	4.25	399	89	7092	0.13

As the chemosensing study towards different ions was carried out in acetonitrile and acetonitrile/water (80:20), the absorption and emission spectra of 4,5-diarylimidazolyl-phenylalanines **3a-d** were also measured in these mixtures at different pH (10^{-6}-10^{-5} M solution). Buffer systems used were sodium acetate 0.1 M/acetic acid 0.1 M for pH= 4, potassium hydrogenphosphate 0.1 M/sodium hydroxide 0.1 M for pH= 7 and sodium hydrogencarbonate 0.05 M/sodium hydroxide 0.1 M for pH= 10. For every compound, there was a bathochromic shift of both wavelengths of maximum absorption and emission on going from acidic to basic pH, probably indicative of a certain degree of deprotonation at the imidazole NH which in turn lead to a higher charge density at the heterocycle that could be delocalised through the system (Figure 1, for phenylalanine **3b** as representative example). However, the increase in pH was accompanied by a decrease of the relative fluorescence quantum yields (except for phenylalanine **3d** which was practically non fluorescent in aqueous solution (Table 3).

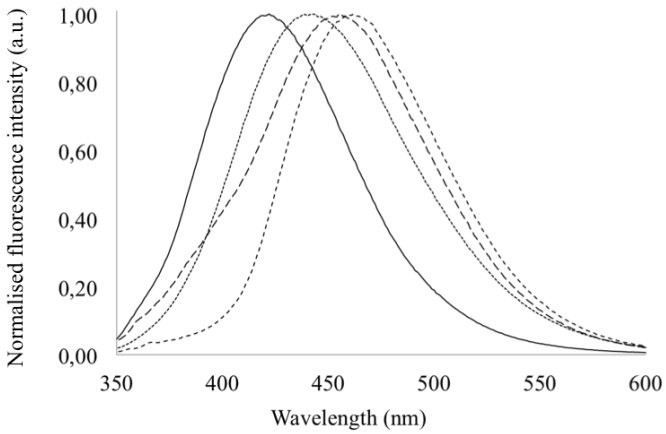


Figure 1. Normalised fluorescence spectra of 4,5-di(thiophen-2'-yl)imidazolyl-phenylalanine **3b** in ACN (—) and ACN/H_2O (80:20) buffered at pH= 4 in (...), pH= 7 (- - -) and pH= 10 (--) ([**3b**]= 6.7×10^{-6} M).

Table 3. UV-visible absorption and emission data for 4,5-diarylimidazolyl-phenylalanines **3a-d** in ACN/H_2O (80:20) buffered at different pH ([**3a-d**]= 10^{-6}-10^{-5} M).

Compound	pH	UV/Vis		Fluorescence			
		λ_{max} (nm)	log ε	λ_{max} (nm)	Stokes' shift (nm)	Stokes' shift (cm^{-1})	Φ_F
3a	4	312	4.22	410	98	7661	0.75
	7	324	4.23	416	92	6826	0.62
	10	329	4.21	422	93	6698	0.55
3b	4	310	4.23	442	132	9634	0.25
	7	325	4.23	455	130	8791	0.20
	10	330	4.23	462	132	8658	0.18
3c	4	295	4.27	396	101	8646	0.46
	7	305	4.29	408	103	8277	0.35
	10	318	4.26	428	110	8082	0.23
3d	4	300	4.26	415	115	9237	0.02
	7	305	4.25	400	96	7895	0.05
	10	309	4.25	397	86	7174	0.04

3.3. Spectrofluorimetric titrations of 4,5-diarylimidazolyl-phenylalanines 3a-d with ions

The modification of phenylalanine through the introduction of extra UV-absorbing and fluorescent heterocycles at its side chain was expected to provide additional binding sites for a variety of ions through the heterocycle donor atoms, as well as improved photophysical properties for the chemosensing studies. It was also intended to assess the influence of the structure in the chemosensing ability of anions and cations.

Evaluation of new 4,5-diarylimidazolyl-phenylalanines **3a-d** as fluorimetric chemosensors was carried out by performing spectrophotometric and spectrofluorimetric titrations in ACN and ACN/H_2O (80:20), in the presence of organic and inorganic anions, and of alkaline, alkaline-earth and transition metal cations (AcO^-, F^-, Cl^-, Br^-, I^-, ClO_4^-, CN^-, NO_3^-, BzO^-, OH^- $H_2PO_4^-$, HSO_4^-, Na^+, K^+, Cs^+, Ag^+, Cu^+, Cu^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Pd^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Cr^{3+}), with biological, environmental and analytical relevance.

A preliminary test was carried out by addition of up to 100 equiv of each ion to the solutions of phenylalanines **3a-d**. No noticeable changes occurred in the colour of the solutions but significant changes were visible in the fluorescence intensity upon interaction with some ions, such as Cu^{2+}, Fe^{3+}, F^- and OH^-.

Therefore, spectrofluorimetric titrations of phenylalanines **3a-d** in acetonitrile with Cu^{2+}, Fe^{3+}, F^- and OH^- were carried out. With Cu^{2+} a decrease of the fluorescence intensity (a chelation enhanced quenching effect, CHEQ) was observed with a complete fluorescence quenching. In Figure 2A, as representative example, is shown the spectrofluorimetric titration of phenylalanine **3a** with Cu^{2+}, where the drastic effect of cation complexation is evident in the band of maximum emission centred at 317 nm, with 10 equiv of cation being sufficient for the complete quenching (Figure 2A). The quenching effect in the presence of Cu^{2+} can be attributed to an energy transfer quenching of the π^* emissive state through low-lying metal-centered unfilled *d*-orbitals [5e]. This result suggests the involvement of the metal ion with donor atoms, the N from the imidazole and the O or S from the pendant furan or thiophene.

With regard to the other ions, with Fe^{3+} a pronounced CHEQ effect was also observed after ion addition (with 15 equiv), accompanied by a red shift of the emission band, whereas with F^- and OH^- an incomplete quenching of fluorescence (ca. 70%, about 20 equiv to achieve the plateau) was seen. In Figures 2 B, C and D are shown the spectrofluorimetric titrations of phenylalanine **3a** with Fe^{3+}, F^- and OH^-, respectively. These anions also induced a quenching of the fluorescence band that could be ascribed to a proton transfer process that yields the deprotonated probe [5b].

As for 4,5-diarylimidazolyl-phenylalanines **3b-d**, the spectrofluorimetric titrations in acetonitrile with Fe^{3+} resulted in a complete quenching with the addition of 180, 280 and 0.35 metal equiv, respectively. Also, titration with both anions induced a similar incomplete fluorescence quenching after addition of 10 equiv of F^- or OH^- (of about 75% for **3b**, 45 % for **3c** and 40% for **3d**). Overall, 4,5-di(furan-2'-yl)imidazolyl-phenylalanine **3a** and 4,5-di(thiophen-2'-yl)imidazolyl-phenylalanine **3b** were the most sensitive towards Cu^{2+} and Fe^{3+}, with phenylalanine **3a** being the most interesting candidate as chemosensor due to the higher fluorescence quantum yield, which is important for maximization of response to analyte in the analysis of very dilute samples. On the other hand, the very low original fluorescence of 4,5-di(pyridin-2'-yl)imidazolyl-phenylalanine **3d** precluded its application as a fluorimetric chemosensor, although it was extremely sensitive to Cu^{2+} and Fe^{3+} with complete quenching after addition of 1.5 and 0.35 equiv of metal, respectively.

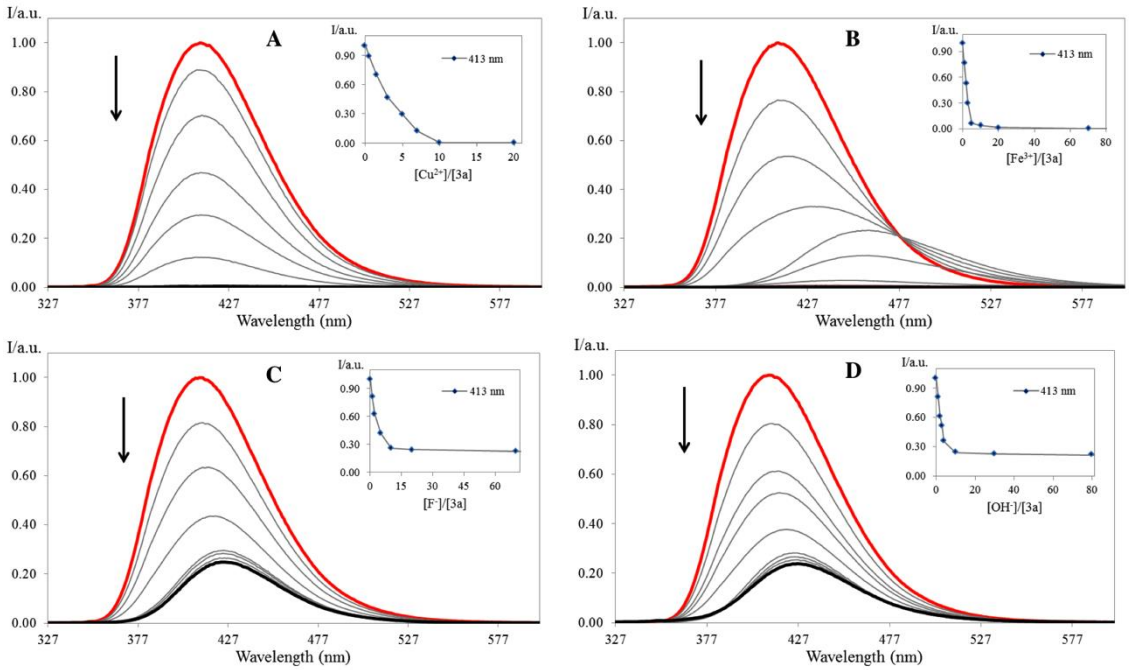


Figure 2. Fluorimetric titrations of 4,5-di(furan-2'-yl)imidazolyl-phenylalanine **3a** with Cu^{2+} (A), Fe^{3+} (B), F^- (C) and OH^- (D) in acetonitrile [λ_{exc} **3a** = 317 nm, [**3a**]= 7.2×10^{-6} M, the cations in the form of perchlorate salts and the anions in the form of tetrabutylammonium salts]. Inset: normalised emission at 413 nm as a function of added ion equivalents.

Having in mind practical applications of compounds **3a-d** in aqueous media, the chemosensory ability was also evaluated in mixtures of acetonitrile and water in varying proportions. The best results, considering a compromise of fluorescence intensity and compound solubility, were obtained in acetonitrile/H_2O (80:20, v/v). The study of the same ions described before in organic aqueous solution lead to lower detection sensitivity since it was necessary to increase the number of equivalents of cation to obtain a similar quenching response, and the fluorescence was not affected in the case of the interaction with $F^-$and OH^-. In the case of Cu^{2+} and Fe^{3+}, higher sensitivity was obtained for the latter cation in aqueous acetonitrile. In Figure 3, it is compared the behaviour of phenylalanines **3a-b** with Fe^{3+} in ACN and ACN/H_2O (80:20) and in Figure 4 it is presented the interaction between phenylalanines **3c-d** with Fe^{3+} in ACN and ACN/H_2O (80:20). In all cases, the interaction with Fe^{3+} resulted in a red shift of the emission band and for phenylalanine **3b** the initial amount of cation added caused an increase of the fluorescence intensity (a chelation enhancement of fluorescence, CHEF effect) which reverted to the quenching as the addition continued.

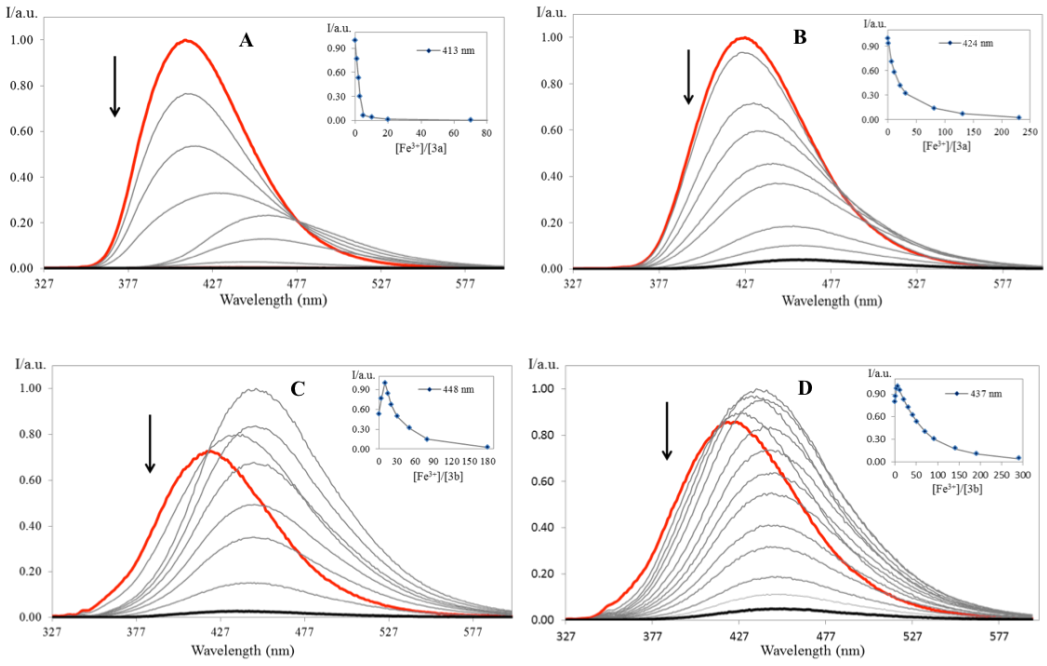


Figure 3. Fluorimetric titrations of 4,5-di(furan-2'-yl)imidazolyl-phenylalanine **3a** with Fe^{3+} in ACN (A) and with Fe^{3+} in ACN/H_2O (80:20, pH 7) (B), and 4,5-di(thiophen-2'-yl)imidazolyl-phenylalanine **3b** with Fe^{3+} in ACN (C) and with Fe^{3+} in ACN/H_2O (80:20, pH 7) (D), [λ_{exc} **3a** = 317 nm, [**3a**]= 7.2×10^{-6} M; λ_{exc} **3b** = 313 nm, [**3b**]= 6.7×10^{-6} M, the cation

in the form of perchlorate salt]. Inset: normalised emission at the wavelength of maximum emission as a function of added ion equivalents.

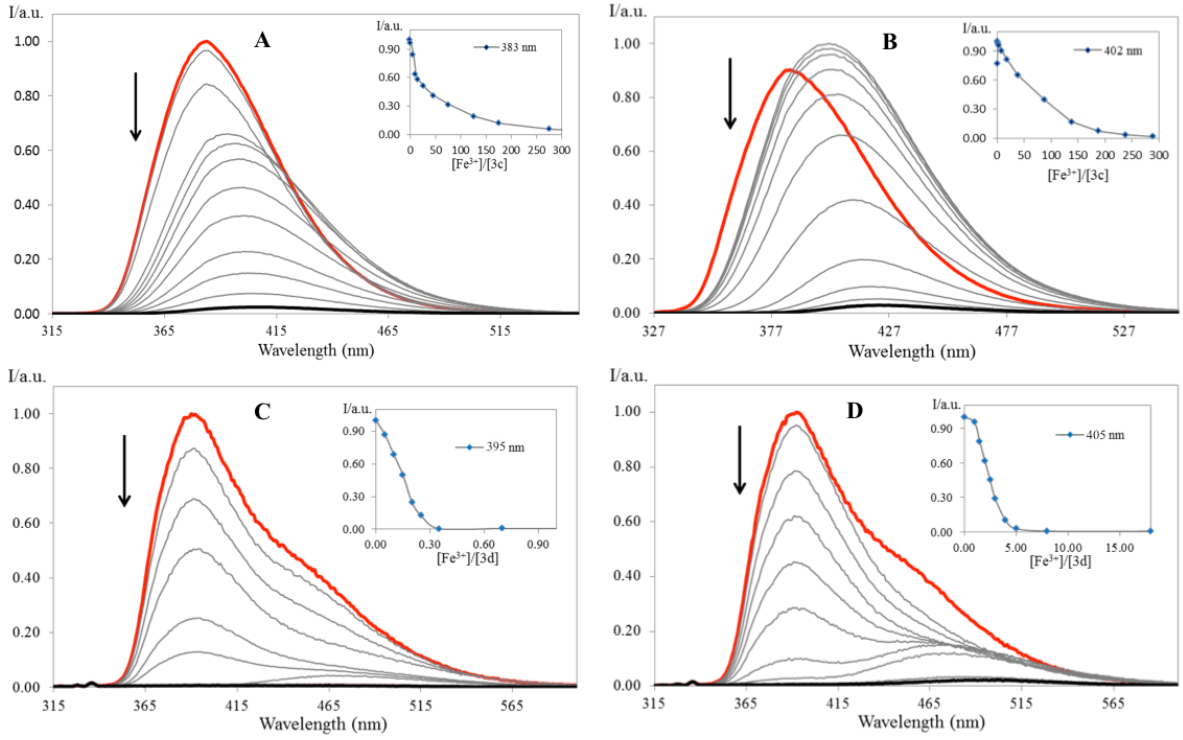


Figure 4. Fluorimetric titrations of 4,5-diphenylimidazolyl-phenylalanine **3c** with Fe^{3+} in ACN (A) and with Fe^{3+} in ACN/H_2O (80:20, pH 7) (B), and 4,5-di(pyridin-2'-yl)imidazolyl-phenylalanine **3d** with Fe^{3+} in ACN (C) and with Fe^{3+} in ACN/H_2O (80:20, pH 7) (D), [λ_{exc} **3c**,**d** = 305 nm, [**3c**]= [**3d**]= 6.8 × 10^{-6} M, the cation in the form of perchlorate salt]. Inset: normalised emission at the wavelength of maximum emission as a function of added ion equivalents.

Previous studies suggested that the free amino acid terminals did not influence significantly the coordination and that it should preferably occur through the heteroatoms at the side chain of the amino acid [12e]. Also, our previous work in synthetic fluorescent amino acids indicated that these amino acids maintain their sensing ability when incorporated into small sequences and, therefore, the resulting peptides displayed sensing ability as well [9a].

3.4. ^{1}H NMR titrations for compound 3b

In order to elucidate the binding mode, ^{1}H NMR titrations were carried out in DMSO-d_6 for compound **3b** with F^- and Cu^{2+}, as representative examples, due to insolubility of compound

3b in deuterated acetonitrile in the required concentration. These titrations revealed that the coordination site involved the imidazole and thiophene, as the signals for these two moieties were more altered upon anion/cation addition (Figures 5 and 6). The signal of the imidazole NH appearing downfield suggested high acidity and strong hydrogen-bonding ability. In more detail, upon addition of F^- only 1 equiv of the ion to ensure complete deprotonation of the imidazole NH and both thiophene rings become equivalent and only one set of proton signals was visible. Deprotonation caused an increase in the electron density in the imidazole ring, which in turn induced a shielding effect on the neighbouring thiophene and phenyl rings owing to through-bond effects. With increasing amounts of F^-, the thiophene protons H3'', H4'' and H5'' shifted upfield as well the phenylalanine protons H2 and H6. The acetyl NH was only disturbed after addition of 5 up to 15 equiv of F^- shifting downfield and becoming broader (Figure 5). Although not shown in the figure, one triplet appeared at lower field (≈16.5 ppm) suggesting the formation of HF_2^- ion, thus providing additional confirmation of the deprotonation of the imidazole NH [5d,e,f].

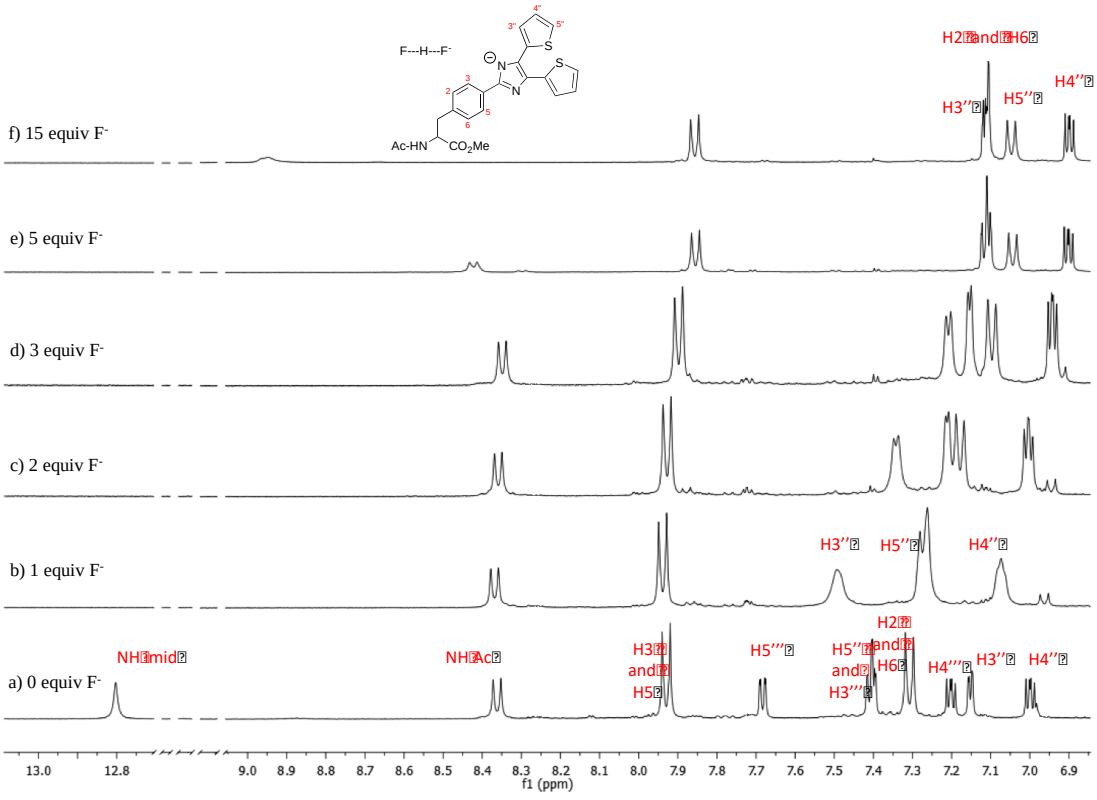


Figure 5. Partial ^{1}H NMR spectra of compound **3b** (1.7×10^{-2} M) in DMSO-d_6 in the absence (a) and the presence of 1.0 (b), 2.0 (c), 3.0 (d), 5.0 (e) and 15.0 (f) equivalents of F^- (in the form of its tetrabutylammonium salt).

As for the titration with Cu^{2+}, there was a considerable broadening of the proton signals, as expected, but similar tendencies were seen in the chemical shifts. Upon addition of only 0.5 equiv of Cu^{2+}, the imidazole NH significantly broadened while the thiophene protons coalesced into only one set of proton signals. With increasing addition of the cation, these protons shifted upfield, but to a lesser extent when compared to the titration with F^- (Figure 6).

Based on the results of the spectrofluorimetric and ^1H NMR titrations, a coordination scheme can be proposed involving the amino acid side chain: the observed chemical shifts are tentatively ascribed to preferential interaction between the anion/cation with one of the imidazole nitrogens and the heteroatom of the pendant heterocyclic moiety (Figures 5 and 6).

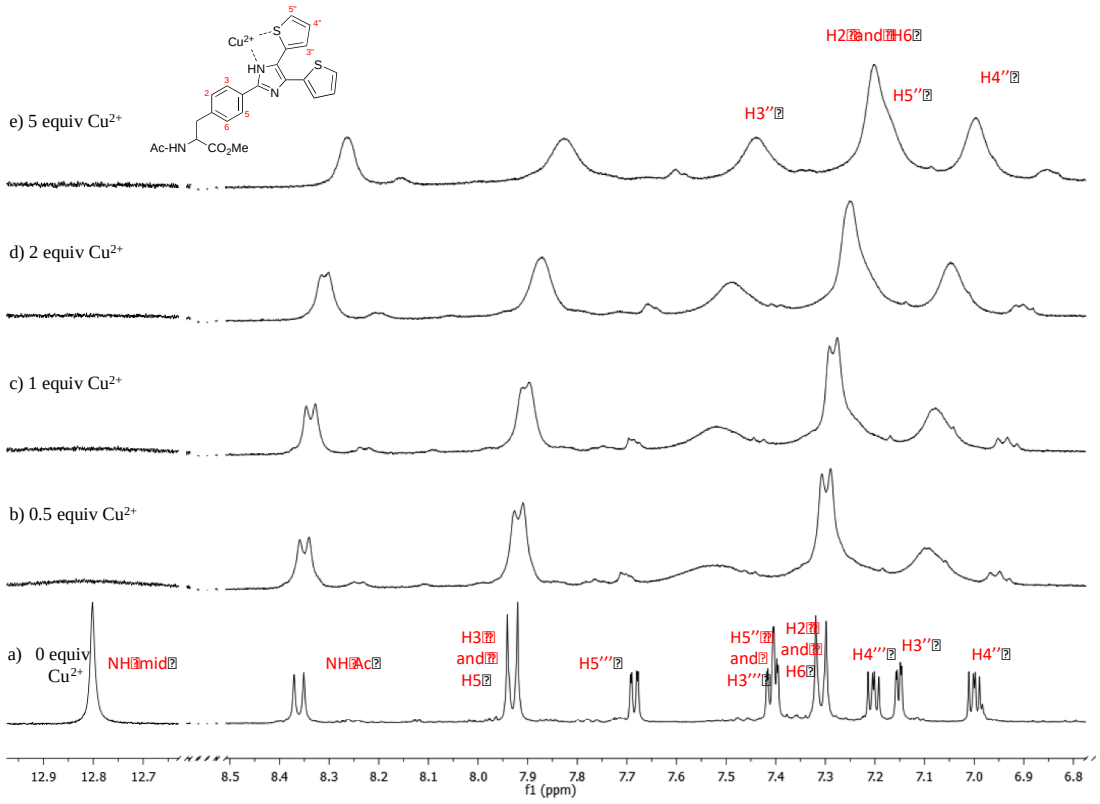


Figure 6. Partial ^1H NMR spectra of compound **3b** (1.7×10^{-2} M) in DMSO-d_6 in the absence (a) and the presence of 0.5 (b), 1.0 (c), 2.0 (d), and 5.0 (e) equivalents of Cu^{2+} (in the form of its perchlorate salt).

The binding stoichiometry of 4,5-diarylimidazolyl-phenylalanines **3a-d** with selected anions/cations and the binding affinity were calculated by HypSpec software from the

spectrofluorimetric titrations in acetonitrile and aqueous acetonitrile, suggesting a 1:1 ligand:anion/metal cation stoichiometry (Table 4).

Table 4. Logarithmic association constants (log K_{ass}) for the interaction of 4,5-diarylimidazolyl-phenylalanines **3a-d** with several anions/cations in acetonitrile (L:M or L:A stoichiometry suggested from HypSpec is 1:1).

Ligand	Ion	log K_{ass} (ACN)	log K_{ass} (ACN/H_2O 80:20)
3a	Cu^{2+}	10.31 ± 0.07	-----
	Fe^{3+}	11.07 ± 0.08	8.38 ± 0.01
	Hg^{2+}	10.23 ± 0.02	9.17 ± 0.03
	Pd^{2+}	10.17 ± 0.09	-----
	F^-	9.24 ± 0.11	-----
3b	Cu^{2+}	11.5 ± 0.23	-----
	Fe^{3+}	11.3 ± 0.12	7.42 ± 0.09
	Hg^{2+}	10.9 ± 0.34	-----
	Pd^{2+}	11.8 ± 0.41	-----
	F^-	7.74 ± 0.31	-----
3c	Cu^{2+}	12.09 ± 0.53	-----
	Fe^{3+}	12.31 ± 0.09	8.25 ± 0.12
	Hg^{2+}	10.27 ± 0.05	-----
	Pd^{2+}	10.44 ± 0.15	-----
	F^-	10.08 ± 0.21	-----
3d	Cu^{2+}	12.25 ± 0.04	-----
	Fe^{3+}	12.92 ± 0.14	9.12 ± 0.02
	Hg^{2+}	11.88 ± 0.03	-----
	Pd^{2+}	11.48 ± 0.07	-----
	F^-	9.44 ± 0.08	-----

The 1:1 stoichiometry of the complex formed between probes **3a-d** and Cu^{2+} was confirmed by spectrofluorimetry by the method of continuous variation and the Job's plot for the

interaction between **3a** and **3b** (as representative examples) and the divalent cation is shown in Figure 7.

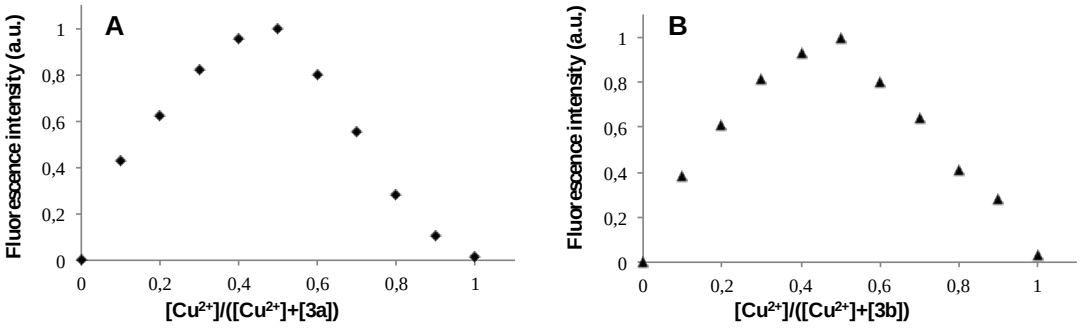


Figure 7. Job's plot for the interaction of **3a** (A) and **3b** (B) with Cu^{2+}, in the form of perchlorate salt, as determined by spectrofluorimetry in acetonitrile at 413 nm for **3a** ([**3a**] + $[Cu^{2+}]$ = 1.0 x 10^{-5} M) and 422 nm for **3b** ([**3b**] + $[Cu^{2+}]$ = 1.0 x 10^{-5} M).

In summary, the advantages of using such unnatural amino acids as sensory units, when compared to natural amino acids, for example, are as follows: the extra heteroatoms at the side chain can exert a cooperative effect for a more efficient binding process and the overall sensing ability; they show enhanced optical response as compounds **3a-c** are much more fluorescent than tryptophan (the most fluorescent natural amino acid with a fluorescence quantum yield of 0.14). This allows higher detection sensitivity, thus lowering detection and quantification limits. Nevertheless, compound **3d** has the same fluorescence quantum yield as tryptophan but it displays longer wavelengths of absorption (excitation) and fluorescence, which is useful for certain biological applications; their intrinsic biological nature and their potential for use in a variety of biological assays based on fluorescence spectroscopy.

4. Conclusions

The novel 4,5-diarylimidazolyl-phenylalanines **3a-d** displayed modest to excellent fluorescence quantum yields (Φ_F = 0.64-0.77 for **3a**; Φ_F = 0.14-0.30 for **3b;** Φ_F = 0.20-0.46 and for **3c** Φ_F = 0.02-0.14) and large Stokes' shifts (between 86 and 119 nm or 6253 and 9099 cm^{-1}) in organic solvents of different character. 4,5-Di(furan-2'-yl)imidazolyl-phenylalanine **3a** displayed the highest relative fluorescence yield in all the solvents and aqueous mixtures. Through spectrofluorimetric titrations with several ions it was found that phenylalanines **3a-d** showed high sensitivity and ability to interact with Cu^{2+} and Fe^{3+} in

ACN but this sensitivity decreased in ACN/H_2O (80:20). The derivatives containing the five-membered heterocycles **3a** (furan) and **3b** (thiophene) required less equivalents to show a complete fluorescence quenching, when compared to derivatives **3c-d**, which can be related to the effect of the electron donor oxygen and sulphur atoms on the overall complexation ability. Due to their emissive character and their cation sensing properties, these heterocyclic amino acids could find application as useful building blocks for peptides with extra functionality due to their UV-active/fluorescent chromophore and complexing capability.

Acknowledgments

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